

Diagnosis of gonorrhoea by culture on a selective medium containing vancomycin, colistin, nystatin and trimethoprim (VCNT)

A comparison with Gram-staining and immunofluorescence

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Culture media containing antibiotics are widely used for the isolation of *Neisseria gonorrhoeae*. The best known are those introduced by Thayer and Martin, who originally used ristocetin and Polymyxin B (Thayer and Martin, 1964), and more recently, and more successfully, vancomycin and colistin (Thayer and Martin, 1966). The main fault with these media is their failure to inhibit *Proteus* species which are found in an important proportion of specimens from the female genital tract. However, *Proteus* species are usually sensitive to trimethoprim, while pathogenic *Neisseriae* are relatively resistant, with minimal inhibitory concentrations (MICs) of more than 8 µg./ml. (Darrell, Garrod, and Waterworth, 1968; Bushby, 1969; Waterworth, 1969). On the basis of these sensitivity results *in vitro*, trimethoprim seemed to deserve trial as a further selective agent for the isolation of gonococci. This paper reports preliminary experiments and an extensive trial, over a period of 2 years in a Venereal Diseases Clinic, of a medium containing vancomycin, colistin, nystatin, and trimethoprim (VCNT). Early results with this medium have been briefly referred to previously (Lancet, 1970; Phillips, 1970).

Methods

PREPARATION OF MEDIUM

VCNT has the following composition: vancomycin 3 µg./ml., colistin methane sulphonate 100 units/ml., nystatin 12.5 units/ml., trimethoprim 5 µg./ml., in Oxoid Blood Agar Base No. 2 (C.M. 271) containing 10 per cent. lysed horse blood. In the earlier part of the

work we used Oxoid Diagnostic Sensitivity Test Agar Base (C.M. 261) (Lancet, 1970) until we found Blood Agar Base more satisfactory.

Stock solutions of vancomycin (3,000 µg./ml.), colistin methane sulphonate (20,000 units/ml.), and trimethoprim (5,000 µg./ml.) are prepared and a 10 per cent. solution of saponin is sterilized by autoclaving at 15 lb. for 15 min. The solutions are then mixed aseptically in the following proportions: vancomycin 10 ml., colistin 50 ml., trimethoprim 10 ml., and saponin 30 ml. Aliquots of 10 ml. of this mixture are stored at -20°C. Nystatin is reconstituted to produce a suspension containing 12,500 units/ml. and is used immediately. Each batch of VCNT medium is made up by preparing to a final volume of 1 litre, Oxoid Blood Agar Base No. 2 cooled to 50°C., to which are added 100 ml. horse blood, 10 ml. VCT saponin mixture, and 10 ml. nystatin suspension. Plates are then poured immediately, and used within 48 hrs.

PRELIMINARY INVESTIGATIONS

Minimum inhibitory concentrations (MICs) of trimethoprim for 365 strains of *N. gonorrhoeae*, and for 63 strains of *Proteus* species, and MICs of vancomycin for 417 strains of *N. gonorrhoeae*, were determined by methods described elsewhere (Phillips, Rimmer, Ridley, Lynn, and Warren, 1970). All the gonococci used in this part of the study were isolated on non-selective medium. Twenty freshly isolated strains of *N. gonorrhoeae* were sub-cultured in parallel on VCNT and the same medium without antibiotics (LBA), which had been found in previous work to yield good growth of gonococci.

CLINIC STUDY

Male patients

Smears of urethral or rectal pus were stained by Gram's method and examined by the Clinic staff. Samples from the urethra, and from the rectum if indicated, were collected by wire loop and inoculated on VCNT and

LBA in one series, and on VCNT alone in a second larger series.

Female patients

Smears of pus from the urethra, cervix, vagina, and rectum were stained by Gram's method, and samples from each site were inoculated on VCNT by the Clinic staff. In the cases of a number of female patients who were thought to be gonorrhoea contacts, smears of pus and films prepared from the cultures after 18 hrs' incubation (at which stage there was usually no macroscopic growth), were stained by immunofluorescence: these investigations are referred to as 'direct' and 'delayed' immunofluorescence (Thin, 1970; Thin, Williams, and Nicol, 1971).

All cultures were immediately incubated at 37°C. in candle-extinction jars, and were examined in the Bacteriology Laboratory after 24 and 48 hrs' incubation. *N. gonorrhoeae* was identified as an oxidase positive organism, with appropriate morphology on Gram staining, that almost invariably grew on VCNT but grew poorly or not at all on nutrient agar (Thayer and Garson, 1965). This method of identification was checked by sugar fermentation reactions of a proportion of the strains, and fermentation reactions were also used to identify the few gonococcal strains that failed to grow on VCNT.

Results

PRELIMINARY STUDY

Table I shows the range of MICs of trimethoprim and vancomycin for *N. gonorrhoeae*, and of trimethoprim for *Proteus* species.

TABLE I *Minimum inhibitory concentrations of trimethoprim and vancomycin for Neisseria gonorrhoeae and Proteus species*

Organism	Antibacterial agent	No. of strains	Minimum inhibitory concentration (µg./ml.)						
			0.4	0.8	1.5	3	6	12	>12
<i>Neisseria gonorrhoeae</i>	Trimethoprim	365	16	5	2	11	37	119	175
	Vancomycin	417	0	11	12	15	62	99	218
<i>Proteus mirabilis</i>	Trimethoprim	48	15	16	3	9	2	1	2
Indole-positive <i>Proteus</i>	Trimethoprim	15	—	8	4	3	—	—	—

TABLE III *Comparison of Gram-stained films with culture on VCNT medium*

Sex	Site	No. of patients examined	No. with evidence of gonorrhoea on either film or culture	Results (per cent.)			Gram-staining
				+	+	—	
Male	Urethra	1,545	518	86	10	4	VCNT culture
	Rectum	66	18	50	28	22	
Female	All sites	2,557	282	68	8	24	

TABLE II *Culture on VCNT and non-selective LBA of urethral pus from 289 males with evidence of gonorrhoea from Gram-stained films*

Culture on:	VCNT	+	+	—	—
	LBA	+	—	+	—
Percentage		68	23.5	1.5	7

Parallel cultures of gonococci and *Proteus* on VCNT and LBA showed that gonococci grew on VCNT, although often somewhat more slowly and sometimes with considerable variation in the size of colonies, but that *Proteus* was almost totally inhibited, with only occasional non-swarming colonies growing in areas of heavy inoculation.

CLINIC STUDY

Male patients

Table II shows the results of parallel cultures on VCNT and LBA of urethral pus containing Gram-negative intracellular diplococci from 289 male patients. In 32 out of 68 samples from which gonococci were isolated from VCNT but not from LBA, there was a heavy growth of organisms on the LBA plate sufficient to obscure possible gonococci. There remain 36 out of 68 in which the organism was isolated from VCNT but not from LBA, not explicable by overgrowth of contaminants. In 7 per cent. of the 289 cases, with Gram-stained films indistinguishable from the rest, gonococci failed to grow on either medium.

Table III shows the results of Gram-stains and cultures on VCNT of 1,545 urethral samples and 66 rectal samples from males. In 518 urethral samples and 18 rectal samples evidence of gonorrhoea was obtained by either or both methods. The proportion of positive cultures from patients with positive films was almost the same as that shown in Table II, again with about 10 per cent. of cases in which specimens containing 'morphological' gonococci in smears failed to grow. In addition gonococci were isolated from 21 patients (4 per cent.) when no gonococci were detected in Gram-stained films.

Female patients

Table III also includes results of examination of Gram-stained films, and of cultures on VCNT, of samples from 2,557 female patients, 282 of whom were found to have evidence, on either criterion, of gonococcal infection. As in males, a similar proportion, 8 per cent., of patients with evidence of gonorrhoea from Gram-stained films had negative culture results, but in almost a quarter of all the cases the diagnosis was made only by culture—that is, gonococci were grown when no gonococci were seen in Gram-stained films.

Table IV shows the results of a comparison of immunofluorescence tests and VCNT culture in the cases of 299 women, 181 of whom had evidence of gonococcal infection by either criterion. Taking all sites, a quarter of the women in Series I had positive immunofluorescence films but negative culture results. Because of this finding, a further study of the delayed immunofluorescence method and culture of samples from 374 more females was made when it was felt that the methods had become more familiar to both the Clinic and Laboratory staffs. Results of this second study are also included in Table IV and show that only 14 per cent. of cases had positive immunofluorescence results with negative cultures. A third, more recent, series has now been completed and the results are also included in Table IV. They show a further decrease of cases with positive immunofluorescence results and negative cultures to 8 per cent. The detailed analysis of results of immunofluorescence tests and culture of samples from the urethra, cervix, vagina, and rectum shows a somewhat larger number of discrepancies with material from the rectum than from other sites. Results of the direct and delayed immunofluorescence methods are very similar except with specimens from the rectum. These detailed results apply to Series I (Table IV, overleaf).

Table V shows the rate of isolation of gonococci from the urethra, cervix, vagina, and rectum in

262 females with positive results to cultures from at least one of these sites.

Table VI analyses similar results, excluding vaginal cultures, in a further 198 females from whom gonococci were isolated and shows the rates of isolation from the three sites separately and in all possible combinations.

Discussion

Since the introduction of the use of trimethoprim in selective media for the isolation of gonococci (*Lancet*, 1970), a series of reports has confirmed its usefulness in inhibiting the growth of those organisms, especially *Proteus* species, that otherwise overgrow and obscure the gonococcus. Our preliminary results with the use of a medium containing 5 µg./ml. trimethoprim in addition to vancomycin and colistin (Phillips, 1970) showed that contaminated samples were more likely to yield a recognizable growth of gonococci on this medium than on any previously available to us. Further experience with VCNT has not changed this conclusion.

In the series of cultures from males with gonorrhoea now reported, the gonococcus was isolated in 24 per cent. from VCNT but not from LBA. This is a higher discrepancy than would be expected, due in part to an unusual degree of contamination of specimens, but also to a paradoxical failure to find gonococci on LBA, not explicable by overgrowth in about half of these cases.

For cultures from females, although we did not make a simultaneous study of VCNT and LBA, a comparison of the present series with an earlier one from St. Thomas' Hospital using non-selective medium (Thin and others, 1971) suggests that the use of VCNT has increased the isolation rate for gonococci by about 10 per cent. by suppressing other organisms. Seth (1970) provided the most striking direct demonstration of the specific value of trimethoprim by selecting a series of samples highly likely to contain *Proteus* and showing that 8 µg./ml. of trimethoprim suppressed the growth of *Proteus* in 57 out of 58 samples, revealing the otherwise undetected gonococcus in seven instances. Riddell and Buck (1970) found that 3 µg./ml. trimethoprim reduced the viable counts of *Proteus* by 10^8 to 10^6 organisms per ml. In the U.S.A., Martin and Lester (1971) reported complete suppression of 39 out of 41 strains of *Proteus* when trimethoprim 5 µg./ml. was incorporated in their new 'Transgrow' medium, and in Oslo Odegaard (1971) reported good results with the same concentration of trimethoprim in a Thayer-Martin type medium.

TABLE IV *Comparison of immediate and delayed immunofluorescence with culture on VCNT of samples from females*

Site	Type of immunofluorescence	No. of samples	Positive in either or both	Results (per cent.)			Immunofluorescence VCNT culture
				+	+	—	
				+	—	+	
Urethra	Direct	208	82	59	29	12	
	Delayed	270	150	62	32	6	
Cervix	Direct	197	96	69	25	6	
	Delayed	253	155	70	26	4	
Vagina	Direct	31	7	57	29	14	
	Delayed	92	52	58	31	11	
Rectum	Direct	19	8	25	63	12	
	Delayed	186	85	47	44	9	
All sites SERIES I	Direct and delayed	299	181	72	25	3	
SERIES II	Delayed only	374	173	79	14	7	
SERIES III	Delayed only	209	65	83	8	9	

TABLE V *Isolation of N. gonorrhoeae from four sites in 262 females with positive cultures on VCNT*

No. of patients	Site	Per cent. with N. gonorrhoeae at this site
259	Cervix	90
93	Vagina	78
262	Urethra	78
201	Rectum	42

TABLE VI *Diagnosis of gonorrhoea from results of VCNT cultures of various combinations of urethral (U), cervical (C), and rectal (R) samples from 198 females with positive cultures at any site*

Taking into consideration results of cultures from	Per cent. patients from whom N. gonorrhoeae was isolated	No. of patients with N. gonorrhoeae at only one site
UCR	100	36
CR	98.5	33
UC	96	28
C	90	25
UR	87	11
U	80	3
R	44	8

It would seem important that there should not be too much trimethoprim in the medium. Seth (1970) based his use of 8 µg./ml. on the finding that 44 strains of *Neisseria gonorrhoeae* all grew on 8 µg./ml. trimethoprim. However, in an examination of about 400 strains, we have found that between 12 and 22 per cent. of gonococci would have MICs of 5 µg./ml. —we cannot be more accurate as our series of antibiotic concentrations for MICs included 3 µg./ml.

and 6 µg./ml. but not 5 µg./ml. In addition, 9 per cent. were inhibited by 3 µg./ml. vancomycin. From these results one would predict that up to 30 per cent. of strains of gonococci might fail to grow on VCNT. The series of parallel cultures of urethral pus from males with gonorrhoea showed that this did not happen: only 1.5 per cent. of strains grew on LBA but not VCNT, and strains in which vancomycin or trimethoprim MICs were below 3 µg./ml. almost invariably grew on VCNT. This further paradox is at present awaiting an explanation. It therefore seems possible that a concentration of trimethoprim of 5 µg./ml. is reasonable in that it will inhibit most strains of *Proteus* without inhibiting the large majority of strains of *N. gonorrhoeae*.

Our failure to isolate gonococci on either medium from samples from 7 per cent. of males with evidence of gonorrhoea on Gram-stained films is disappointing. We are examining clinic and laboratory procedure, which we think may be at fault, but it is possible that LBA is not as good for the isolation of gonococci as it is for their subsequent culture. Riddell and Buck (1970) compared chocolate agar with our formulation of LBA and found them equally effective, although they did not study directly the relatively simple problem of the culture of gonococci from males. It is noteworthy that Schroeter and Pazin (1970) claim a 100 per cent. yield for Thayer-Martin medium.

As expected, culture on VCNT was much more reliable than Gram-staining of smears in the diagnosis of gonorrhoea in females. Almost one-quarter of the 282 infected women were diagnosed on the

basis of culture alone (Table III). Eight per cent. of specimens had positive results to Gram-stained films with organisms indistinguishable from those in the rest, but culture results were negative. As a proportion of all females with positive Gram-stained films, this is higher than in males, and may have a similar but as yet unknown explanation.

When culture on VCNT was compared with immunofluorescence staining by either direct or delayed methods, there were initially almost 25 per cent. of women who had positive results to smears but negative culture results. With increasing experience this proportion fell to 14 per cent. and then to 8 per cent. In the earlier study already referred to, Thin and others (1971) found positive results by delayed immunofluorescence in samples from 125 women and by direct immunofluorescence in 119; and positive culture results on non-selective medium in 85—that is 24 per cent. with positive immunofluorescence and negative culture results. As Reyn (1969) has pointed out, the results in this type of investigation will depend on the medium, the techniques, and the specificity of the antiserum. We used Difco antiserum characterized by Thin (1970) in the way he described, including the staining of control slides which always gave satisfactory results. It would be naïve to suppose that the method is *always* specific for *N. gonorrhoeae* and it is possible that some of our discrepancies are due to non-specific fluorescence or cross-reactions. However, in a few cases, organisms in every way characteristic of gonococci by immunofluorescence fail to grow, just as do organisms typical of gonococci in Gram-stained smears of samples from both men and women. For these reasons, it is not surprising that with somewhat different techniques some workers have found that delayed immunofluorescence yields more positive results than culture on media of various types (Fry and Wilkinson 1964); Lucas, Price, and Thayer, 1967), that some have found the results of immunofluorescence and culture on selective media to correspond very closely (Lind, 1969), and that others have had more positive results from culture than from immunofluorescence (Martin, Peacock, and Thayer, 1965; McGill, Moffett, Masterton, and Schofield, 1969). In addition to this, Schmale, Martin, and Domesick (1969) found that cultures were negative at the first examination in 6 to 8 per cent. of infected females, a factor that we did not take into account. The addition of trimethoprim to the selective medium has not resolved these discrepancies. We agree with Lind (1969) that it would seem wise to use both culture on the best selective medium and the delayed immunofluorescence method whenever possible.

Our results of cultures from different sites in females suggest that, if the maximum number of isolations of gonococci is to be made, specimens from the urethra and cervix, and also from the rectum, should be cultured. However, if only cervical and rectal samples had been cultured, only three patients out of 198 would not have been diagnosed by this method. This is in agreement with the findings of Schmale and others (1969), as is our finding that vaginal samples yielded positive cultures in 78 per cent. of cases, with the important difference that because VCNT contains trimethoprim no cultures had to be rejected because they were overgrown by 'spreaders'.

Summary

A medium containing vancomycin, colistin, nystatin, and trimethoprim (VCNT) has been used in a Venereal Diseases Clinic for the isolation of *N. gonorrhoeae*. The suppression of growth of organisms other than gonococci, particularly *Proteus*, was good.

In samples from males, gonococci were paradoxically isolated more frequently from VCNT than from a similar medium without antibiotics, even in the absence of overgrowth by contaminants, in spite of the fact that a few gonococci had MICs for vancomycin and trimethoprim below the concentrations in the medium. 4 per cent. of urethral samples from males had negative Gram-stain results but positive cultures, while about 10 per cent. of samples containing 'morphological' gonococci in Gram-stained films did not yield the organism on culture. About one-quarter of rectal samples from males had positive Gram-stained films and negative culture results, and the same proportion had negative films and positive cultures.

In samples from females, specimens showing gonococci in Gram-stained films did not grow in 8 per cent. of all those with evidence of gonorrhoea, but in a quarter of the patients, gonococci grew when none had been seen in films. Immunofluorescence staining of smears was more efficient than Gram-staining and, although at first 25 per cent. of females had positive immunofluorescence results but negative cultures, in later series this figure was reduced to 14 per cent., and then to 8 per cent., possibly as a result of greater experience in the Clinic and in the Laboratory.

The proportion of cases with negative immunofluorescence results and positive cultures rose from 3 to 7 per cent. and then to 9 per cent. in these three studies. These results suggest that both immunofluorescence testing and culture on VCNT should be

used to reach a diagnosis in as many female patients as possible.

It was also found that, in females with evidence of gonorrhoea on culture, samples from the cervix and rectum together were positive in 98.5 per cent., while cultures from the vagina were positive in 78 per cent.

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Diagnostic de la gonococcie par culture utilisant un milieu sélectif contenant vancomycine, colistine, nystatine et triméthoprime (VCNT). Comparaison avec la coloration de Gram et l'immuno-fluorescence

SOMMAIRE

Dans une clinique vénéréologique, on a utilisé pour l'isolement de *N. gonorrhoeae* un milieu contenant vancomycine, colistine, nystatine et triméthoprime (VCNT). L'inhibition d'autres organismes que le gonocoque, *Proteus* en particulier, fut satisfaisante.

Chez les hommes, paradoxalement, on isola plus fréquemment des gonocoques dans le milieu VCNT que dans un milieu similaire ne contenant pas d'antibiotiques; ceci même en l'absence du développement concurrentiel de contaminants et en dépit du fait que la concentration inhibitrice minimale, pour un petit nombre de gonocoques, était, vis-à-vis de la vancomycine et de la triméthoprime, en dessous des concentrations de ces produits dans le milieu. 4 pour cent des prélèvements urétraux chez l'homme donnèrent des résultats négatifs pour le Gram et positifs pour les cultures alors que les cultures étaient négatives pour environ 10 pour cent de cas où les lames montraient des organismes ayant la morphologie du gonocoque. Pour environ un quart des prélèvements rectaux chez l'homme, la coloration de Gram était positive et la culture négative; la même proportion de cas fut trouvée négative sur lame et positive en culture.

Chez les femmes, les prélèvements montrant des gonocoques sur lame ne poussèrent pas dans 8 pour cent de tous les cas de gonococcie prouvée, mais chez un quart des malades la culture fut positive alors que les lames ne montraient pas de gonocoques. La coloration des étalements par immuno-fluorescence fut plus efficace que la coloration de Gram et, quoiqu'au début 25 pour cent des femmes étaient positives avec l'immuno-fluorescence mais négatives en culture, dans les dernières séries cette proportion fut réduite à 14, puis à 8 pour cent; probablement parce que la Clinique et le Laboratoire avaient acquis une plus grande expérience.

Dans ces trois études, la proportion des cas donnant des résultats négatifs à l'immuno-fluorescence et des cultures positives passa de 3 à 7, puis à 9 pour cent. Ces résultats font penser que les épreuves d'immuno-fluorescence et la culture au VCNT doivent être toutes les deux utilisées chez le plus de malades femmes possible pour permettre d'arriver au diagnostic.

Il a aussi été constaté que, chez les femmes présentant une gonococcie prouvée par culture, les prélèvements du col et du rectum donnaient ensemble une positivité de 98,5 pour cent, alors que les cultures à partir du vagin étaient positives dans 78 pour cent.